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Original Research

Presence of enterotoxigenic Clostridium *perfringens* in foods of animal origin, Guwahati, India

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Abstract
Aim: To isolate *Clostridium perfringens* from foods of animal origin and detect the presence of enterotoxin gene in the isolates in Guwahati, India.
Methods: This study was carried out from August 2010 to February 2011. A total of 400 food samples comprising 50 each of chevon, pork, fish, beef and liquid raw milk, curd, pasteurized milk powder, pasteurized liquid milk and ice cream were screened for the presence of *Clostridium perfringens*. A total of 33 food samples were tested positive for the presence of the organism.
Results: Among the 33 isolates of *C. perfringens*, 5(15.15%) were found positive for enterotoxin gene (*cpe*) by Polymerase chain reaction. The result showed that those foods which tested positive for enterotoxigenic *C.perfringens* had potential to cause food poisoning. Even those foods which tested negative for the presence of enterotoxigenic *C. perfringens* could be contaminated from the environment due to poor handling of foods pre- and post-processing.
Conclusion: Therefore, hygiene should be maintained at all levels of food preparation in order to prevent food borne illness caused by such organism.

INTRODUCTION

Members of the genus *Clostridium* are anaerobic, spore-forming, rod-shaped gram-positive bacteria. They exist as free-living bacteria as well as pathogens that infect both humans and animals. Most pathogenic *Clostridium* species are known to produce toxins, which are responsible for a wide range of diseases [1].

Clostridium perfringens is commonly found in the gastrointestinal tract of both humans and other animals, as well as in soil and sewage. It has been shown to be a cause of human diseases such as gas gangrene (Clostridial myonecrosis), food poisoning and necrotizing enterocolitis of infants [2, 3]. It is different from many other clostridia in that it is non- motile and, *in-vitro*, forms spores only in specialized culture media [4].

Clostridium perfringens can grow between 15^{o}C and 50^{o}C with an optimum of 45^{o}C for most strains. The generation time (Gt) for most strains at temperatures between 33^{o}C and 49^{o}C is below 20 min, while Gt of 8 min has also been reported [5]. The organism can produce over 13 different toxins although each bacterium only produces a subset of these toxins [6].

Clostridium perfringens is classified into five types (A–E) on the basis of their ability to produce major lethal toxins (alpha, beta, epsilon and iota) [7, 8]. The alpha (α) toxin, encoded by the *cpa* gene, is a phosholipase-C and is produced by all *C. perfringens* types A, B, C, D and E [9] and has been shown to contribute to the pathogenesis of gas gangrene via different mechanisms

[10].

A minority of *C. perfringens* strains produce a *C. perfringens* enterotoxin (CPE), which is responsible for the symptoms of common *C. perfringens* food poisoning. Enterotoxigenic *C. perfringens* have also been associated with sporadic cases of diarrhoea and with some cases of sudden infant death syndrome [11-14]. Different meats have been frequently reported as the most common food vehicles [1, 15-18].

The *C. perfringens* enterotoxin (CPE), produced during sporulation of vegetative cells in the host intestine and formation of the spore coat layers, has a unique four step membrane action that binds to receptors on intestinal epithelial cells and exerts its intestinal action resulting in the characteristic diarrhoea and abdominal cramping symptoms associated with *C. perfringens* food poisoning [19].

Food-borne diseases present a growing health problem worldwide and over 200 different diseases are known to be transmitted by food and food products of animal origin. *Clostridium perfringens* is responsible for one of the most common types of food poisoning. Symptoms associated with *C. perfringens* are caused by an enterotoxin which is produced during sporulation of the organism in the small intestine following ingestion of large numbers of vegetative cells of enterotoxin-positive *C. perfringens* [20]. Enterotoxin encoded by *cpe* gene is the only toxin that is not secreted from vegetative cells but produced during sporulation [21].

Despite several works in the area regarding food borne pathogens, there were no previous works in the present study area which showed the importance of *Clostridium perfringens* as a food borne risk. The organism being ubiquitous has the potential to contaminate food even after processing. Hence the study was significant to the area as it could give background information for subsequent studies in the area. Hence the present study was carried out to detect the presence of *C. perfringens* in foods of animal origin and characterise the isolates for the presence of enterotoxin gene.

MATERIALS AND METHODS

Collection of Samples

A total of 400 samples were collected. Raw/fresh meat and milk products (approximately 50g) and liquid milk (approximately 100 ml) were collected aseptically in UV irradiated polyethylene sachets. They were immediately brought to the laboratory under ice for microbiological analysis. The types of food samples collected are shown in Table 1.

Isolation and Identification

For isolation of C. *perfringens* homogenates for meat were prepared by cutting 25 g of meat aseptically into small pieces and homogenizing with 225 ml peptone dilution fluid (0.1% peptone water, pH 7.0 ± 0.1, autoclaved at 121^{o}C for 15 minutes). Similarly, homogenates from curd, milk powder and ice cream were prepared. The homogenates and other food samples (raw milk and milk products) were inoculated deep into Robertson's cooked meat broth (RCMB; Hi-Media Laboratories Pvt. Ltd., Mumbai) in contact with the meat. The inoculated RCMB tubes were placed in water bath for a period of 10-15 minutes at 80^{o}C to eliminate the non-spore forming aerobic bacteria. Finally, the RCMB tubes were incubated in anaerobic jar at 44^{o}C for 48 h. For selective isolation, an agar overlay technique was used in sterile petridish. Shahidi-Ferguson-Perfringens (SFP) agar was used as selective agar. 0.1 ml RCMB-enriched inoculum was taken in a sterile petridish and 20 ml of sterile SFP medium was added with thorough mixing. It was allowed to solidify for 5-10 minutes and again 10 ml of SFP medium was overlaid. The petridish was then incubated at 37^{o}C for 24 h. Black colonies suspected for *C. perfringens* were picked up with an inoculation loop and subjected to gram staining, motility and biochemical tests for confirmation as per [22].

Table 1. Sources and distribution of foods of animal origin and their products

Type of food sample collected	Place of collection	Number of samples collected
Chevon	Butchers' shops	50
Chicken	Butchers' shops	50
Beef	Butchers' shops	50
Pork	Butchers' shops	50
Fish	Local retail shops	50
Raw milk	Livestock farms	50
Pasteurised milk	Retail outlets	25
Milk powder	Retail outlets	25
Ice-cream	Retail outlets	25
Curd	Retail outlets	25
Total		400

Detection of Enterotoxin gene in isolates by Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed to detect enterotoxin gene by using primers specific to *cpe* according to [23]
i.e.,F:5′-GGAGATGGTTGGATATTAGG-3′;
R:5′-GGACCAGCAGGTTGTAGATA-3′(Hysel, India Pvt. Ltd., New Delhi). Predicted size of amplified product for the specific oligonucleotide primers was 233 bp.

Template was prepared using Hipura Genomic DNA

Purification spin kit (Hi-Media Laboratories Pvt. Ltd., Mumbai). An overnight Brain Heart Infusion (BHI) broth culture was harvested by centrifugation for 2 minutes at 13, 000 rpm. The pellet was then re-suspended in 1.5 ml lysozyme and incubated for 30 minutes at 37°C. Then the sample was treated with 20 µl proteinase K (20 mg/ml) and 20 µl of RNase and incubated at room temperature for 5 minutes. It was then treated with 200 µl lysis solution, vortexed for few seconds and incubated at 55°C for 10 minutes to produce lysate. After incubation, 200 µl ethanol was added to the lysates. The suspension was shaken vigorously, transferred to spin column and centrifuged at 10, 000 rpm for 1 minute. The flow-through liquid was discarded after each centrifugation and the spin column transferred to another tube. Then 500 µl prewash solution was added to the spin column and centrifuged at 10,000 rpm for 1 minute. This was followed by adding 500 µl wash solution to the spin column and centrifuging it at 13, 000 rpm for 3 minutes. The spin column was transferred to another tube and centrifuged for 1 minute to dry the column. Finally 200 µl of elution buffer was added directly into the column without spilling to the sides. This was incubated for 5 minutes at room temperature and then centrifuged at 10,000 rpm for 1 min to elute the DNA. The eluate was stored at –20°C to be used as DNA template.

The amplification of bacterial DNA for detection of *cpe* was performed in thermocycler (BioRad, USA) in 25µl volume containing 12.5 µl of 2X master mix (4 mM $MgCl_2$, 0.4mM of each dNTPs, 0.05 U/ml of Taq DNA polymerase, 150 mM Tris-HCL PCR buffer, MBI Fermentas), 0.5 µM primers and 1 µl of the template DNA. The conditions for PCR were 94°C for 3 min for initial denaturation of DNA within the sample followed by 30 cycles of 94°C for 30s (denaturation), 55°C for 1 min (primer annealing), 72°C for 45s (DNA extension) and a final extension at 72°C for 5 min. The amplified PCR product was kept at 4°C till further analysis.

The amplification product of 233 bp was confirmed by electrophoresis in 1 % agarose gel having ethidium bromide at a concentration of 0.5 mg/ml using 100 bp DNA ladder as marker.

RESULTS

In the present study, isolation of *Clostridium perfringens* was attempted from various food samples of animal origin. The isolates were identified as *C. perfringens* on the basis of their cultural, morphological and biochemical characteristics. Polymerase chain reaction (PCR) was used to detect the presence of enterotoxin gene in the isolates.

Prevalence of *Clostridium perfringens* in foods of animal origin

A total of 400 food samples, which comprised of raw meat (250) of different food animals and milk (50) and milk products (100), were screened for isolation of *C. perfringens*. Bacteriological examination revealed presence of the organism in 33 (8.25%) out of the 400 samples as depicted in Table 2.

Table 2. Isolation rate of *C. perfringens* from different foods of animal origin

Type of food sample collected	Number of samples screened	No. of samples positive for *C. perfringens* (%)
Chevon	50	14(28.00)
Chicken	50	6(12.00)
Pork	50	5(10.00)
Fish	50	3(6.00)
Raw milk	50	5(10.00)
Beef	50	0(0.00)
Milk products	100	0(0.00)
Total	400	33 (11.20)

The highest prevalence of *C. perfringens* was found in chevon 14 (28.00%) followed by chicken 6(12.00%), pork 5(10.00%), milk 5 (10.00%) and fish 3(6.00%) as depicted in Table-2. However, beef and milk products (curd, ice-cream, milk powder and pasteurized milk) did not reveal the presence of the organism.

Detection of enterotoxin gene in *Clostridium perfringens* isolates by Polymerase Chain Reaction

In the PCR assay, altogether 33 isolates were studied for the presence of *cpe*. Five (15.15%) showed amplification products of 233 bp for enterotoxin gene, similar to those of the positive control.

Out of 33 isolates from various foods tested, four chevon and one chicken isolate revealed the presence of *C. perfringens* enterotoxin (*cpe*) gene (Table 3).

Table 3. Prevalence of enterotoxigenic *Clostridium perfringens* in foods of animal origin

Type of food sample	No. of isolates obtained	No. of isolates possessing *cpe (%)*
Chevon	14	4
Chicken	6	1
Pork	5	0
Fish	3	0
Raw milk	5	0
Total	33	5(15.15%)

DISCUSSION

Out of the total 400 samples, 33 yielded *C. perfringens* which were confirmed by morphological, cultural and

biochemical characteristics. All the meat samples except beef, and milk showed presence of *C. perfringens* ranging from 6 to 28 percent (Table 2), while beef and processed milk products (curd, pasteurized milk, milk powder and ice-cream) consistently tested negative for the presence of *C. perfringens*.

The overall prevalence of *C. perfringens* (8.25%) is low as compared to similar works done in other places [24 -26]. This low prevalence could be attributed to heat-shocking of the medium in the present study in order to kill non-spore-forming aerobic bacteria. This is well supported by [27] who reported *C. perfringens* at a rate of 2 percent and 29 percent in heat-shocked and non-heat shocked samples, respectively. It was suggested that vegetative cells were killed by heat shocking.

The highest prevalence was recorded in chevon (28 %). This finding was close to the report by previous worker [24] who reported C. *perfringens* in chevon with prevalence between 35 to 75 percent. The highest prevalence in chevon in the present study could be due to increased chance of contamination of the carcass during evisceration as the organism is natural flora of the gut. Moreover, the slaughtering is performed on the ground which might have increased the chance of contamination of the carcass from soil.

Out of the 33 *C. perfringens* isolates, 28(11.20%) were recovered from meat. This finding is in agreement with the works of [28, 29], who recorded 10.5 to 35 percent *C. perfringens* from retail market meat. Five (10.00%) pork samples yielded *C. perfringens*. This is similar with works of [30] who isolated *C. perfringens* from 10 percent of unprocessed raw pork. However, in the present study, no C. *perfringens* isolate could be recovered from beef. The area from where beef samples were collected conducted evisceration while hanging the animal. This might have contributed in the avoidance of contamination of the carcass from the intestinal contents and soil.

Sea foods could be contaminated by *C. perfringens* through contaminated water bodies, which in turn get infected due to excretion of the organism in faeces of various carrier animals [31]. (Ampratwum, Acquaintance, Personal communication, October 2010) recorded isolation of *C. perfringens* from 48.1 percent and 20 percent, respectively, of fresh water fish. However, in this study, only 6 percent of such samples tested positive for the organism. Those fish might have come from water bodies which could have been contaminated by faeces of man or animals, or the water used for washing fish might be contaminated.

Out of the 150 samples of raw milk and milk products, only raw milk samples yielded *C. perfringens* with prevalence of 10 percent. The present observations on prevalence of C. *perfringens* in milk samples were similar to the findings of [32] who observed presence of *C. perfringens* in 9.3 percent of milk samples. However, previous work in same geographical area (Mehtaz, Research Associate, Personal Communication, November 2010) recorded prevalence of the organism ranging from 20 to 47.4 percent on milk. This apparent difference in the prevalence rate could be due to improvement in milk handling and distribution system. All the milk products tested were negative for presence of *C. perfringens* which might suggest improvement in handling, processing and storage of milk products. The presence of *C. perfringens* in milk might be due to contamination from faeces of carrier animals. This might show that unhygienic milking practices contributed to contamination of milk. The milk could also be contaminated due to improper handling.

In the present study, *C. perfringens* was more frequently isolated from meat (11.20%) than milk and milk products (3.33%). This might be because of the fact that *C. perfringens* lacks the ability to produce 13 of the 20 essential amino acids and is, therefore, associated with protein-rich foods and 75 percent of the food-borne outbreaks can be traced to meat and meat products [33, 34].

The PCR assay was performed on the 33 isolates of *C. perfringens* from foods of animal origin. Out of these, five (15.15%) were found positive for *cpe*, four from chevon and one from chicken (Table 3 and Figure 1). The same observation was made by [26]. Enterotoxigenic strains of *C. perfringens* were estimated to represent less than five percent of global *C. perfringens* isolates (35) accounting for their rare isolation and detection in food. [27] could detect one percent enterotoxigenic *C. perfringens* from 900 retail foods of animal origin while [36] did not detect any enterotoxigenic *C. perfringens* from retail foods of animal and non-animal origin.

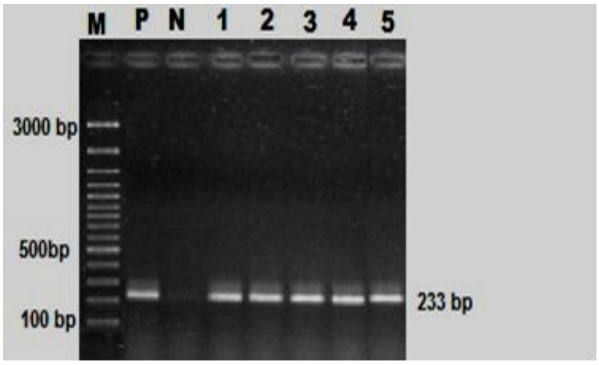

Fig 1. Detection of cpe in Clostridium perfringens isolates by PCR

LANE M: 100 bp DNA LADDER

LANE P: Positive control
LANE N: Negative control
LANE 1-5: Isolates tested positive

Enterotoxin-positive strains occur in low numbers in co-existence with large numbers of *cpe*-negative *C. perfringens*. Therefore, detecting enterotoxigenic *C. perfringens* after conventional cultivation and picking a couple of colonies may yield *cpe* negative result. This is because of the fact that *cpe*-positive stains are dominated by the *cpe*-negative strains in the culture [37]. This may result in failure in picking those positive strains for PCR test. However, the detection of enterotoxin gene in *C. perfringens* isolates could indicate that such foods might be the potential sources of food poisoning.

The study indicated that chicken meat and chevon could be the possible sources of food poisoning. Even though the gene was not detected in the other foods, *cpe*-positive *C. perfringens* strains that are present in the environment may be transmitted to food by post processing contamination. When favourable conditions occur, the strains can multiply in the food and cause food poisoning. Thus efforts are of utmost importance to detect enterotoxigenic *C. perfringens* directly from raw as well as processed cooked foods, which enables to detect the source of contamination in the food.

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